4

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 743 084 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
20.11.1996 Bulletin 1996/47

(21) Application number: 96107794.8

(22) Date of filing: 15.05.1996

(51) Int. Cl.^{6}: **B01D 15/00**, B01J 20/24, B01J 20/32, B01D 15/08, A61M 1/36, B01J 20/22

(84) Designated Contracting States:
AT BE CH DE ES FR GB LI NL SE

(30) Priority: 16.05.1995 IT TO950387

(71) Applicant: BELLCO S.p.A.
I-41037 Mirandola (IT)

(72) Inventors:
- Tetta, Ciro
  41037 Mirandola (IT)
- Gervasio, Renzo
  10132 Torino (IT)
- Wratten, Mary Lou
  41036 Medolla (IT)

(74) Representative: Plebani, Rinaldo et al
c/o Studio Torta,
Via Viotti 9
10121 Torino (IT)

(54) Dialysis filter/reactor for capturing endotoxins, and relative fabrication method

(57) A dialysis filter/reactor including at least a hollow fiber made of ultrafiltering cellulose-based material and inside which polymyxin is immobilized; the polymyxin being covalently bonded to the cellulose fiber by activation prior to immobilization and using sodium periodate as an oxidant, and by stabilization subsequent to immobilization and using sodium borohydride as a reducer; the activating step being performed for a relatively long period of time (1.5 h) with a low concentration (2.5 gr/l) of sodium periodate, and by circulating an aqueous solution of sodium periodate inside the fibers of the filter at a very high specific flow rate (1500 mm^3/minute/mm^2).

HYDRAULIC PERMEABILITY
activated filters vs nonactivated filter

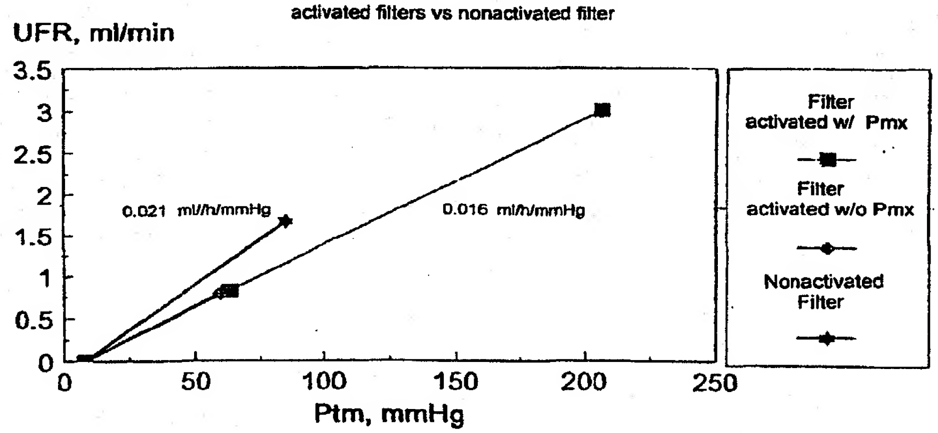

Fig. 3

EP 0 743 084 A1

Printed by Rank Xerox (UK) Business Services
2.13.8/3.4

Description

The present invention relates to a dialysis filter for extracorporeal treatment of human whole blood or plasma, and which at the same time acts as a reactor for capturing any endotoxins in the blood or plasma. More specifically, the present invention relates to a dialysis filter/reactor wherein endotoxins are captured by an active peptide (polymyxin) immobilized on the walls of the filter contacting the blood or plasma, and which is used for renal support and/or treatment of patients suffering from septic shock.

The present invention also relates to a method of fabricating such a filter/reactor, and more specifically to a method of immobilizing polymyxin inside hollow cellulose fibers with no alteration in the physical-mechanical characteristics of the fibers.

Despite extensive use of antibiotics, septic syndromes still remain a major cause of disease and death, and, during such syndromes, it is vital not only to fight the cause of the infection, but also to provide the patient with general systemic support, particularly renal support. Septic syndromes, also known as "septic shock", are characterized by a drastic reduction in systemic vascular resistance, myocardial depression, and diffused organ failure, the main cause of which lies in infectious agents (generally bacteria) releasing the endotoxins on their cell walls into the patient's bloodstream. One possible supportive therapy is to inject the patient with an antibiotic comprising a peptide - polymyxin (PMX-B) - capable of bonding the endotoxins. This, however, can only be done in small doses and with extreme care, on account of the highly toxic nature of polymyxin at renal level.

One solution proposed and tested to overcome this drawback [W.G.Cheadle et al. - SURGERY 1991; 110:785-92] is to treat the blood of septic shock patients extracorporeally by hemoperfusion through columns/filters in which polymyxin is covalently bonded to insoluble polystyrene fibers (PMX-F) according to a Japanese process developed by TORAY. Despite commercial application, wherein hemoperfusion is performed through a cartridge comprising a number of polystyrene pellets on which the polymyxin is immobilized, this system presents several drawbacks, foremost of which is the fact that it only applies in the main to plasma.

As stated, in fact, in the above and other publications [W.G.Cheadle et al. - SURGERY 1991; 110:785-92], when applied to whole blood, extensive thrombosis phenomena of the PMX-F matrix occur unless accompanied by parallel supportive therapy using heparin or other anticoagulants, which, however, are particularly dangerous and may result in extensive hemorrhaging in the case of septic shock patients.

German Patent DE-A-4113602, on the other hand, relates to the removal of endotoxins by extracorporeal hemo- or plasmaperfusion using columns or capsules, wherein a matrix defined by flat porous cellulose-based sheets immobilizes an endotoxin-capturing agent comprising polyethylimine, which is immobilized on the cellulose support either by adsorption or indirectly by ionic bonding using supports comprising anionic cellulose derivatives. Polymyxin-based systems, however, would appear more effective.

Italian Patent Applications n. 67212-A/80 and 67023-A/81 relate to methods of immobilizing peptides comprising hepatic enzymes, in particular Glutathione-S-Transferase, inside hollow cellulose fibers of the type used in hemodialysis filters. This is done by forming covalent bonds between the amino groups of the enzyme and the aldehyde groups of the cellulose - such as Schiff bases - by activating the aldehyde groups with an oxidant, and subsequently stabilizing the bond with a reducer.

Both these patents highlight the importance of selecting the right process conditions for satisfactory results to be obtained (immobilization of a considerable amount of enzyme, long-term immobilization stability, and no change in the cellulose structure, permeability or mechanical strength of the fibers). In particular, the action of the oxidant (sodium periodate) must be carefully timed, in that, over and above 70-80 minutes, the fibers are so oxidized as to be unusable as filters.

It is an object of the present invention to provide a system for the systemic support, in particular renal support, and/or treatment of patients affected with septic shock, and which is based on simultaneously removing endotoxins and any dialyzable harmful substances extracorporeally and directly from whole blood. It is a further object of the present invention to enable the use, with only minor changes, of conventional currently marketed dialysis equipment, and to provide a highly reliable, relatively low-cost system by which absolutely no potentially harmful substances are released into the patient's bloodstream.

According to the present invention, there is provided a dialysis filter/reactor comprising at least a hollow fiber made of ultrafiltering, cellulose-based material; characterized in that polymyxin is immobilized on the inner lateral surface of said fiber, and is covalently bonded to the cellulose fiber by Schiff bases; said filter/reactor being equally capable of treating, and removing any endotoxins from, aqueous solutions, whole blood or plasma.

Such a dialysis filter/reactor provides simultaneously for extracorporeal ultrafiltration and dialysis of circulating whole blood or plasma, and for capturing and removing any endotoxins in said blood or plasma. According to a further aspect of the present invention, such a filter is fabricated using a method characterized by comprising:

- an activating step wherein a dialysis filter of hollow cellulose-based fibers is activated by circulating a solution of an oxidant comprising sodium periodate inside said hollow fibers;

- an immobilizing step, subsequent to said activating step, wherein a solution with a relatively high concentration of polymyxin is circulated inside said hollow fibers; and
- a stabilizing step, subsequent to said immobilizing step, wherein a solution of a reducing agent comprising sodium borohydride is circulated inside said hollow fibers.

In particular, the activating step is performed using a solution with a relatively low concentration of sodium periodate, and by feeding said solution inside the hollow cellulose fibers at a relatively high specific flow rate for a relatively long period of time. For example, a sterile aqueous solution of sodium periodate with a concentration of 2.5 gr/l is fed through the hollow fibers at a specific flow rate of at least 1500 mm^3/minute/mm^2 of fiber section for over 70 minutes. Preferably, the activating step comprises recirculating said oxidizing solution of sodium periodate through said hollow fibers for 1.5 hours at ambient temperature.

The activating step is followed by a neutralizing step wherein any oxidant residue is neutralized by circulating a sterile aqueous solution of 20% by volume of glycerol through said fibers. Preferably, the immobilizing and stabilizing steps are also performed by feeding the respective said solutions through the hollow fibers at the same specific flow rate as for the activating step.

According to a further characteristic of the present invention, the immobilizing step is performed by feeding through the fibers an aqueous solution of polymyxin with a concentration of at least 3 gr/l in a 0.1 M bicarbonate solution, at a temperature ranging between 0 and 4°C, and by recirculating said solution for at least 12 hours.

In practice, polymyxin is highly satisfactorily immobilized inside hollow cellulose dialysis fibers, and with no impairment in the physical-mechanical characteristics of the fibers, by applying, albeit with a few substantial variations as regards process parameter values, substantially the same method traditionally used to immobilize hepatic enzymes on hollow fibers of the same type.

It is important to stress that, nowhere in existing literature, was a specialist in this particular field given to understand the possibility of safely and effectively immobilizing polymyxin on hollow cellulose hemodialysis fibers without affecting the other properties of the fibers. Indeed, known polymyxin immobilizing techniques deal with entirely different types of supports (polystyrene) and are clearly inapplicable to cellulose materials. When using a cellulose support, existing literature has always clearly discouraged the use of polymyxin, and advocated the use of other substances as endotoxin bonding agents.

Finally, known methods of immobilizing hepatic enzymes on cellulose fibers are first of all applied to a particular class of peptides with physical-chemical characteristics differing widely from those of polymyxin. Moreover, experiments conducted of known methods of immobilizing enzymes on cellulose have shown the impossibility of determining beforehand the feasibility of the process using another peptide. That is, whereas the possibility of achieving a given immobilization is indeed predictable, there is absolutely no guarantee of also maintaining the physical-mechanical characteristics of the support, owing to the impossibility of predicting the operating parameters required to effectively immobilize the desired substance.

In the case of the present invention, for example, extensive testing showed that, to satisfactorily immobilize polymyxin, activation could not be achieved using existing hepatic enzyme techniques, and that recourse would have to be made to prolonged fiber-oxidant contact times, even falling within a range explicitly ruled out in existing literature as being capable of irreparably damaging the structure of cellulose fibers. The method according to the present invention, in fact, employs contact times well in excess of the 70 minutes considered maximum by the known state of the art, and, again with no assistance from existing literature, compensates prolonged contact time with very high (i.e. "fast") specific flow rates - equal to over three times the maximum rates in existing literature - and relatively low oxidant concentrations (though still within the accepted limits).

A dialysis filter/reactor in accordance with the present invention is therefore characterized by comprising a bundle of hollow fibers made of cellulose-based ultrafiltering material, preferably CUPROPHAN™, and on the inner lateral surface of which polymyxin is immobilized stably and unreleasably in use; and at the same time presents substantially the same hydraulic permeability as a normal dialysis filter of cellulose fiber with the same physical-mechanical characteristics.

Tests have also shown that a dialysis filter/reactor in accordance with the present invention provides for removing over 90% of the endotoxins present in a stream of solution or plasma or whole blood contaminated with 50 EU/ml of endotoxins.

The immobilizing step is normally preceded by a conditioning step wherein the fibers are conditioned by feeding through them an 8.4 pH bicarbonate solution. Similarly, the activating step is preceded by a wash step using sterile distilled water, in the course of which, the cellulose fibers absorb water to enable the activating solution to penetrate inside the micropores of the fibers by straightforward diffusion. The final stabilizing step is as usual, and comprises circulating a sterile aqueous solution of sodium borohydride with a concentration of 1 gr/l through the filter fibers for at least 2 hours.

Before being fed through the fibers, all the solutions used in the method according to the present invention are fed through a depyrogenating filter under a sterile hood. Moreover, by virtue of merely feeding and partly or fully recirculat-

ing various solutions inside the fibers, the entire method according to the invention may be applied to a normal dialysis filter ready for use, and possibly already installed in a known dialysis machine or specially constructed hydraulic circuit.

A number of non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying drawings, in which:

Figures 1 and 2 show the calibration curves used to test endotoxin removal from an aqueous solution and plasma;
Figure 3 shows the hydraulic permeability characteristics of dialysis filters with and without the treatment according to the present invention.

EXAMPLE 1

- Polymyxin immobilization

Three identical filters, each comprising 100 hydraulically parallel hollow CUPROPHAN™ fibers with an inside diameter of 200 μm (transverse through surface of each filter 3.14 mm^2), are fitted in parallel to a hydraulic test circuit including a reservoir, a pump, and an HFTO2 depyrogenating filter; and each filter is fitted in series to a single-pass line connected to the reservoir and presenting a recirculating bypass controlled by a Klemmer valve.
The filters are then treated as described below, the reservoir being filled with the various solutions, which are circulated through the filters at a rate of 5 ml/minute unless otherwise specified, and the solution, when recirculated, being fed back to the reservoir for further passage through the filters:

1. With the recirculating bypasses closed (single-pass), the filters are washed in parallel with 400 ml of filtered, sterile distilled water.
2. The circuit is emptied and the line filled with 150 ml of a 2.5 gr/l solution of $NaIO_4$, which is recirculated inside the filters for 1.5 hours.
3. The filters are washed with 800 ml of filtered sterile water.
4. The oxidant residue is neutralized with 300 ml of a 20% by volume aqueous solution of glycerol, 90% of which is supplied in a single pass, and 10% of which is recirculated.
5. The filters are conditioned with 60 ml of an 8.4 pH aqueous solution of $NaHCO_3$, 10% of which is recirculated for 5 minutes.
6. The circuit is emptied; the first filter is filled with bicarbonate; and the valves are closed to isolate the relative circuit branch, so that the first filter constitutes the "blank" to which subsequent testing is referred.
7. The lines of the other two filters are filled with 60 ml of an aqueous solution of 3 gr/l of polymyxin in a 0.1 M bicarbonate solution; and the solution, maintained at a temperature of 0 to 4°C, is recirculated through the filters for 12 hours.
8. The recirculated solution is collected; a 1 gr/l concentration of sodium borohydride is added to the recirculated solution in a sterile vessel; and the resulting solution is recirculated through the filters for 2 hours.
9. The filters are washed with 800 ml of a 7.4 pH buffer solution, of which 80% is supplied in a single pass, 10% is recirculated for 30 minutes, and the remaining 10% is again supplied in a single pass.

EXAMPLE 2

- Endotoxin absorption

35 ml of a solution of 50 EU/ml of endotoxin in sterile water and apyrogen are prepared, and 10 ml of blood is incubated with 2 μg/ml of endotoxin (25000 EU/ml); an aliquot of the solution at the initial concentration is stored in a refrigerator; an aliquot of blood not incubated with endotoxin (basal) and an aliquot of blood containing 25000 EU/ml of endotoxin are centrifuged, and the surnatant stored in a refrigerator. Using the same circuit as in Example 1, the endotoxin solution is fed through one of the two filters treated with polymyxin (first sample), and the blood incubated with endotoxin is fed through the second (second sample). The filtered endotoxin solution and incubated blood samples are then collected; the incubated blood sample is centrifuged, and the surnatant (plasma) is collected and stored in a refrigerator. The filtered samples of endotoxin solution and blood (centrifuged plasma) at the initial concentrations are then COATEST analyzed graphically against sterile distilled water and apyrogen, and against the plasma derived from the blood not incubated with endotoxin. The calibration curves are shown in Figures 1 and 2.
The test results are shown in Tables 1 and 2.

TABLE 1

sample	dilut.	A 405 nm	real conc. EU/ml	rem. %
initial	1:100	1.117	40	98.5
final 1st sample	-	1.304	0.55	
final 1st sample	1:10	0.157	0.6	

TABLE 2

sample	dilut.	A 405 nm	real conc. EU/ml	rem. %
initial	1:20,000	1.8175	25,000	96.7
final	1: 1,000	1.934	825	

As shown clearly in Tables 1 and 2, over 90% of the endotoxin is removed from both the solution and whole blood. Moreover, in the whole blood test, no coagulation occurred for the first 4 hours. Subsequently, coagulation occurred gradually, with occlusion of the pores, but only very slowly.

EXAMPLE 3

- Membrane damage test

A standard hydraulic permeability test (ultrafiltration versus transmembrane pressure) was conducted of three filters - a blank, sample 1, sample 2 - and of a fourth nontreated, i.e. nonactivated, filter. The results are given in Figure 3, which shows no significant change in permeability.
At the end of the test, each filter was fed with a concentrated BLUE DEXTRAN™ solution (2,000,000 DALTON) and ultrafiltration was enforced. If any of the membranes had been damaged, the colouring agent would have filtered through with the ultrafiltrate. In this particular case, all the ultrafiltrates remained perfectly transparent, indicating absolutely no damage of the membranes treated with polymyxin, even after use. The filters were then washed with deionized water, each was fed with a concentrated COMASSIE BLUE™ solution (861 DALTON), and ultrafiltration was enforced. None of the membranes passed the colouring agent, thus indicating no change in the sieving properties of the membranes treated with polymyxin, even after use (and even using whole blood).
As all the membranes treated with polymyxin were stained blue, the test was repeated using a membrane of HEMOPHAN™, which is known to present amino groups bonded to the cellulose chains. When treated with COMASSIE BLUE™, this membrane also presented a permanent blue colour, which is due to the interaction, known in literature, of the colouring agent molecules with the available protein cation groups. The colouring of the filters treated with polymyxin therefore confirms immobilization of the polymyxin and the extent to which this remains stable even after use.

Claims

1. A dialysis filter/reactor comprising at least a hollow fiber made of ultrafiltering, cellulose-based material; characterized in that polymyxin is immobilized on the inner lateral surface of said fiber, and is covalently bonded to the cellulose fiber by Schiff bases; said filter/reactor being equally capable of treating, and removing any endotoxins from, aqueous solutions, whole blood or plasma.

2. A dialysis filter/reactor as claimed in Claim 1, characterized by presenting substantially the same hydraulic permeability as a normal dialysis filter of hollow cellulose fiber with the same physical-mechanical characteristics.

3. A dialysis filter/reactor as claimed in Claim 1 or 2, characterized by being capable of removing over 90% of the endotoxins present in a stream of solution or plasma or whole blood contaminated with 50 EU/ml of endotoxins.

4. A dialysis filter/reactor as claimed in any one of the foregoing Claims, characterized in that said hollow cellulose fibers are made of CUPROPHAN™.

5. A method of fabricating a dialysis filter/reactor for extracorporeal ultrafiltration and dialysis of circulating whole blood or plasma, and for capturing and removing any endotoxins in said blood or plasma; said method being characterized by comprising:

- an activating step wherein a dialysis filter of hollow cellulose-based fibers is activated by circulating a solution of an oxidant comprising sodium periodate inside said hollow fibers;
- an immobilizing step, subsequent to said activating step, wherein a solution with a relatively high concentration of polymyxin is circulated inside said hollow fibers; and
- a stabilizing step, subsequent to said immobilizing step, wherein a solution of a reducing agent comprising sodium borohydride is circulated inside said hollow fibers.

6. A method as claimed in Claim 5, characterized in that at least said activating step is performed using a said solution of sodium periodate with a relatively low concentration, and by feeding said solution inside the hollow cellulose fibers at a relatively high specific flow rate for a relatively long period of time.

7. A method as claimed in Claim 6, characterized in that the activating step is performed using a sterile aqueous solution of sodium periodate with a concentration of 2.5 gr/l, and which is fed through the hollow fibers at a specific flow rate of at least 1500 mm^3/minute/mm^2 of fiber section for over 70 minutes.

8. A method as claimed in Claim 6 or 7, characterized in that said activating step comprises recirculating said oxidizing solution of sodium periodate through said hollow fibers for 1.5 hours at ambient temperature.

9. A method as claimed in one of the foregoing Claims from 5 to 8, characterized in that said activating step is followed by a neutralizing step wherein any oxidant residue is neutralized by circulating a sterile aqueous solution of 20% by volume of glycerol through said fibers.

10. A method as claimed in one of the foregoing Claims from 5 to 9, characterized in that said immobilizing and stabilizing steps are performed by feeding the respective said solutions through the hollow fibers at the same specific flow rate as for the activating step.

11. A method as claimed in one of the foregoing Claims from 5 to 10, characterized in that said immobilizing step is performed by feeding through the fibers an aqueous solution of polymyxin with a concentration of at least 3 gr/l in a 0.1 M bicarbonate solution, at a temperature ranging between 0 and 4°C, and by recirculating said solution for at least 12 hours.

12. A method as claimed in Claim 11, characterized in that the immobilizing step is preceded by a conditioning step wherein the fibers are conditioned by feeding through them an 8.4 pH solution of bicarbonate.

13. A method as claimed in one of the foregoing Claims from 5 to 12, characterized in that said stabilizing step comprises circulating through said fibers a sterile aqueous solution of sodium borohydride with a concentration of 1 gr/l for at least 2 hours.

14. A method as claimed in one of the foregoing Claims from 5 to 13, characterized in that, before being fed through said fibers, all said solutions are fed through a depyrogenating filter.

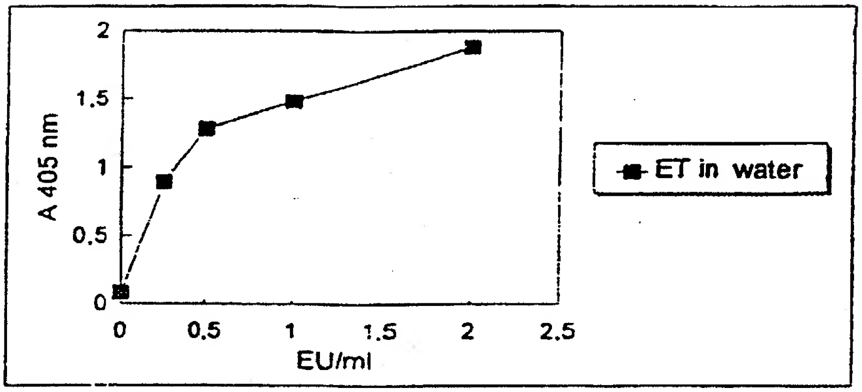
2
1,5
1
0,5
0
A 405 nm
0
0,5
1
1,5
2
2,5
EU/ml
ET in water

Fig. 1

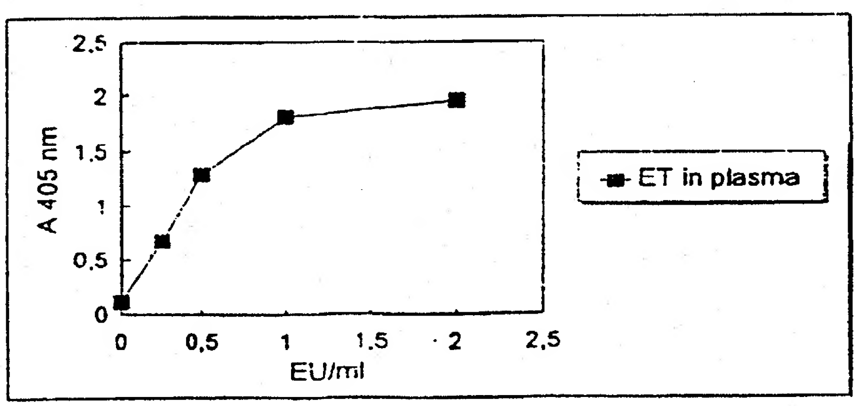
2,5
2
1,5
1
0,5
0
A 405 nm
0
0,5
1
1,5
2
2,5
EU/ml
ET in plasma

Fig. 2

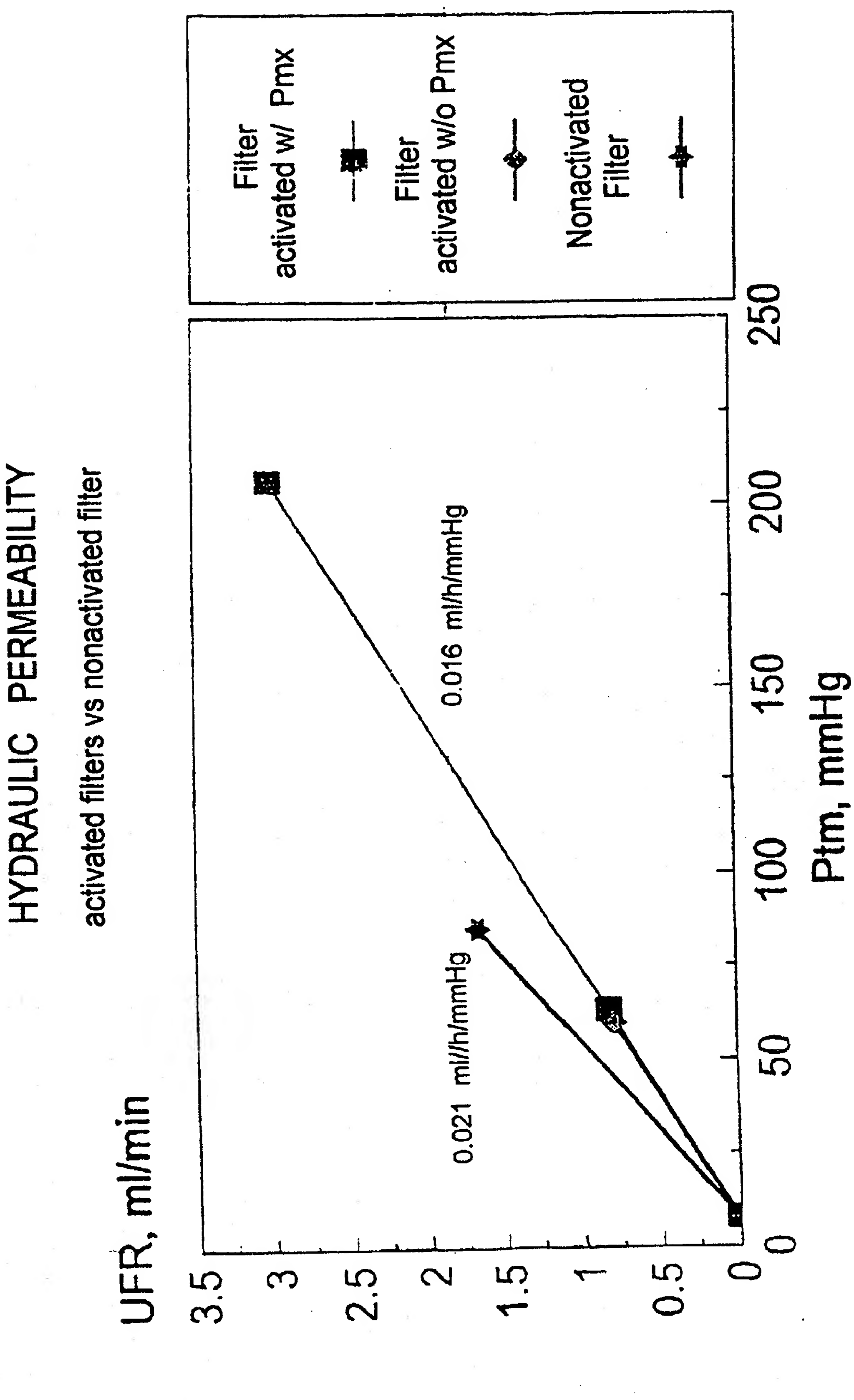
HYDRAULIC PERMEABILITY
activated filters vs nonactivated filter
UFR, ml/min
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
Ptm, mmHg
0.021 ml/h/mmHg
0.016 ml/h/mmHg
Filter activated w/ Pmx
Filter activated w/o Pmx
Nonactivated Filter

Fig. 3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 10 7794

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y A	EP-A-0 034 304 (SORIN BIOMEDICA SPA) 26 August 1981 * page 1, paragraph 1 * * page 2, paragraph 1 * * page 3, paragraph 2 - page 4, paragraph 3 * * page 5, paragraph 2 - page 10, paragraph 1 * * page 14, paragraph 1 * * claims 1,22 * ---	1,2,4-6, 9,12 7,8,13	B01D15/00 B01J20/24 B01J20/32 B01D15/08 A61M1/36 B01J20/22
Y	EP-A-0 328 256 (OWENS CORNING FIBERGLASS CORP) 16 August 1989 * page 1 - page 2, line 28 * * figure 1 * ---	1,2,4-6, 9,12	
A	US-A-4 637 880 (HALBERT SEYMOUR P) 20 January 1987 * column 2, line 64 - column 3, line 17 * * column 4, line 50-61 * * column 5, line 8-15 * ---	1-4	**TECHNICAL FIELDS SEARCHED (Int.Cl.6)**
A	US-A-4 661 260 (KODAMA MASASHI ET AL) 28 April 1987 * abstract * ---	1	B01D B01J A61M
A	EP-A-0 424 672 (KURITA WATER IND LTD) 2 May 1991 * abstract * ---	1	
A	DE-A-41 13 602 (FALKENHAGEN DIETER DR SC MED ;MITZNER STEFFEN DR MED (DE); SCHNEID) 29 October 1992 * abstract * --- -/--	1	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	30 July 1996	Bichlmayer, K-P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 10 7794

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	EP-A-0 312 104 (TANABE SEIYAKU CO ;DAICEL CHEM (JP)) 19 April 1989 * page 2, line 20-48 * * page 3, line 35-44 * ---	1	
A	US-A-3 947 352 (CUATRECASAS PEDRO ET AL) 30 March 1976 * abstract; figure 2 * -----	5	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	30 July 1996	Bichlmayer, K-P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)